

nature genetics

volume 14 no. 4

december 1996

Docket No.: PF-0356-4 CPA
USSN: 09/840,787
Exhibit E

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diagnostics
and genomics**

**Rieger
syndrome**

**QTLs and
epistasis**

**A BRCA1-
binding
protein**



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Cover art: Ken Krattenmaker

Nature Genetics (ISSN 1061-4036) is published monthly by Nature Publishing Co., headquartered at 345 Park Avenue South, New York, NY 10010, which is owned by Nature America Inc., a subsidiary of Macmillan Magazines Ltd, of London. Editorial Office: Nature Genetics, 545 National Press Building, Washington DC 20045. Telephone (202) 626 2513, Fax (202) 626 0970, e-mail natgen@naturedc.com. North American Advertising: Nature Genetics, 345 Park Avenue South, New York, NY 10010. Telephone (212) 726 9200, Fax (212) 696 9606. European Advertising: Nature Genetics, Porters South, Crinan Street, London N1 9XW, UK. Telephone (0171) 833 4000, Fax (0171) 843 4596. New subscriptions, renewals, changes of address, back issues and all customer service questions should be addressed to: Nature Genetics Subscription Dept, 345 Park Avenue South, New York, NY 10010, USA. Telephone (212) 726 9200, Fax (212) 696 9606. Outside North America: Nature Genetics, Macmillan Magazines Ltd, Porters South, Crinan Street, London N1 9XW, UK. Telephone (0171) 833 4000, Fax (0171) 843 4596. Annual subscription rates: US/Canada: US\$495 (institutional/corporate), US\$195 (individual making personal payment), Canada add 7% for GST, BN: 14091 1595 RT; UK: £350 (institutional/corporate), £175 (individual making personal payment). Japan: Contact Nature Japan, K.K., Shin-Mitsuke Bldg. 4F, 3-6 Ichigaya Tamachi, Shinjuku-ku, Tokyo 162. Reprints: Nature Genetics Reprints Dept, 345 Park Avenue South, New York, NY 10010, USA. Telephone (212) 726 9200. Periodicals postage paid at New York, NY and at additional mailing offices.

US MAILS: Send address changes to Nature Genetics Subscription Dept, 345 Park Avenue South, New York, NY 10010. Executive Officers of Nature America, Inc.: Nicholas Byam Shaw, Chairman of the Board; Mary Waltham, President; Edward Vais, Secretary-Treasurer. Published in Japan by Nature Japan KK, Shin-Mitsuke Building, 3F, 3-6 Ichigaya Tamachi, Shinjuku-ku, Tokyo 162, Japan. Printed in the USA by Cadmus Journal Services. ©1996 Nature America, Inc.

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Use of a cDNA microarray to analyse gene expression patterns in human cancer

Joseph DeRisi¹*, Lolita Penland² & Patrick O. Brown² (Group 1); Michael L. Bittner³*, Paul S. Meltzer³, Michael Ray³, Yidong Chen³, Yan A. Su³ & Jeffrey M. Trent³ (Group 2)

The development and progression of cancer¹⁻³ and the experimental reversal of tumorigenicity^{4,5} are accompanied by complex changes in patterns of gene expression. Microarrays of cDNA provide a powerful tool for studying these complex phenomena⁶⁻⁸. The tumorigenic properties of a human melanoma cell line, UACC-903, can be suppressed by introduction of a normal human chromosome 6, resulting in a reduction of growth rate, restoration of contact inhibition, and suppression of both soft agar clonogenicity and tumorigenicity in nude mice^{4,5,9}. We used a high density microarray of 1,161 DNA elements to search for differences in gene expression associated with tumour suppression in this system. Fluorescent probes for hybridization were derived from two sources of cellular mRNA [UACC-903 and UACC-903(+6)] which were labelled with different fluors to provide a direct and internally controlled comparison of the mRNA levels corresponding to each arrayed gene. The fluorescence signals representing hybridization to each arrayed gene were analysed to determine the relative abundance in the two samples of mRNAs corresponding to each gene. Previously unrecognized alterations in the expression of specific genes provide leads for further investigation of the genetic basis of the tumorigenic phenotype of these cells.

DNA microarrays, containing 1,161 total elements, including 870 different cDNAs and controls⁹⁻¹¹ (see Methods), were printed robotically onto a glass microscope slide in four quadrants covering an area of about 1 cm² (Fig. 1). We prepared fluorescent cDNA probes using total poly (A)⁺ mRNA from UACC-903 cells and UACC-903(+6) cells by labelling with a green and red fluor, respectively. A mixture of the two fluorescently labelled probes was hybridized to the DNA microarray. This comparative hybridization method, coupled with the doping of synthetic standards and an estimation of statistically significant deviation for local background variance allowed a direct and quantitative comparison of the relative abundance of individual DNA sequences in this complex sample⁶⁻⁸. We added a set of synthetic poly (A)⁺-tailed 'mRNAs' to the purified mRNA from each cell line as internal standards to assist in quantitation and estimation of experimental variation introduced during labelling and reading. Targets complementary to these standards were included, in duplicate, on the microarray. Based on these standards, mRNA species comprising 1:10,000 of the mass of the poly (A)⁺ RNA could readily be detected.

In a representative two-colour fluorescent scan of all 1,161 arrayed elements (Fig. 2a), the green spots corre-

spond to genes preferentially expressed in the tumorigenic UACC-903 cell line, and the reddish spots correspond to genes preferentially expressed in the non-tumorigenic UACC-903(+6) cell line. Genes expressed at approximately equal levels in the two cell lines appear yellow or brown. A portion of the array at higher magnification highlights the diverse pattern of differential expression observed (Fig. 2b). In Fig. 2c, rectangles corresponding to specific array elements are coloured to reproduce the hue and intensity of the fluorescent signal at each element. The hybridization signals from a duplicated set of genes are shown juxtaposed, to illustrate the reproducibility of the hybridization signals for each gene.

To address the possibility that an apparent difference in expression might result from experimental variables unrelated to the difference in chromosomal composition between the two cell lines, we examined the variance in expression for 90 'housekeeping' genes. We selected these genes based on the assumption that they would not be differentially expressed between the two cell lines. The averaged red/green ratio for this subset of genes was 1.13. The averaged red/green ratio for the set of five internal standards was 0.97 ($n = 10$). The variability in the expression level of the housekeeping genes probably overestimates the experimental variability in measuring differential expression. As a conservative standard, an absolute fluorescent signal (red or green) with an intensity greater than that observed at the control array elements containing total human genomic DNA was considered to represent specific hybridization. Gene-specific hybridization was therefore only considered significantly different between samples if the following two criteria were met: i) the signal intensity (green or red) exceeded this threshold; and ii) the logarithm of the red/green fluorescence signal ratio differed by ≥ 3 S.D. from the mean logarithm of this ratio for the 'housekeeping' gene panel (that is, ratios < 0.52 or > 2.4).

By these criteria, mRNA levels for 15/870 (1.7%) genes were significantly diminished, while the mRNA levels for 63/870 (7.3%) genes were significantly increased in association with suppression of tumorigenicity by introduction of chromosome 6. To test the reliability of microarray hybridization results in identifying differentially expressed genes, we analysed 16 genes by northern analysis. In each case, the results of northern analysis corroborated the differential gene expression identified by microarray hybridization (Fig. 3).

Significant differences in expression between these two cell lines identified several genes as candidates for determining features of the tumorigenic phenotype of the melanoma cells. For example, among the genes detected with significantly higher expression (> 10 -fold) in the tumorigenic cells was the human brown locus protein (TRP1/melanoma antigen gp75). This is the most abundant glycoprotein in melanocytic cells and a critical melanosome membrane protein^{12,13}. Additionally, its expression is reduced when melanoma cell lines are induced to differentiate by treatment with HMBA^{12,13}. Also expressed at a significantly higher level was a spliced variant of the mRNA encoding myelin PLP/DM20. This is widely expressed in neural crest derived cells in early development and has been suggested to play a role in cell-cell signaling during development¹⁴.

A significant number of the genes whose mRNA lev-

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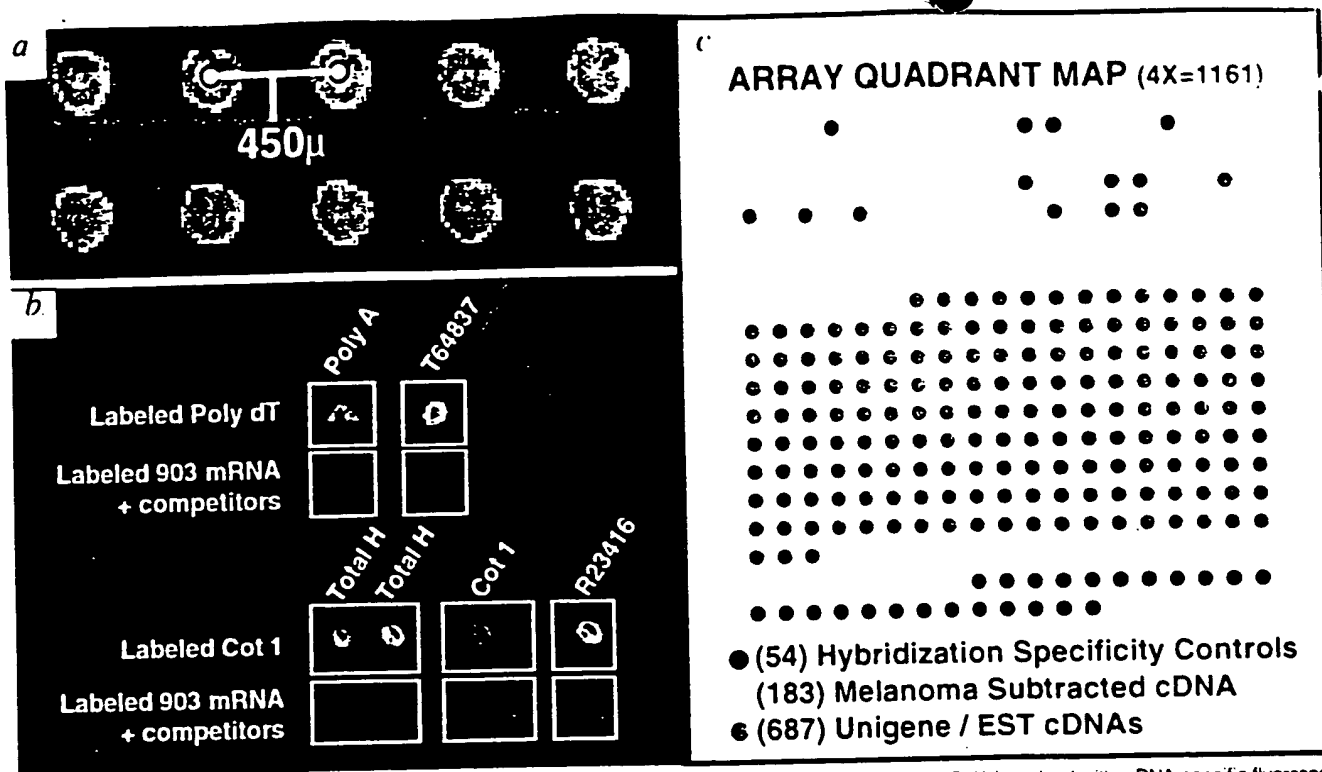


Fig. 1 Properties of cDNA microarrays. **a**, A fluorescent scan of DNA printed onto a poly-lysine coated slide. The DNA is stained with a DNA-specific fluorescent dye, YOYO. The center-to-center spacing of adjacent spots is 450 μ , allowing the potential for up to 10,000 spots/2.54 X 7.62 cm microscope slide. **b**, Efficient blocking of hybridization to DNA repeats. Hybridization of fluorescein-labelled poly (dT)* to arrays in the absence of competitor produces strong hybridization to immobilized poly (dA)* as well as to some cDNAs, such as the EST T64827 shown. Rhodamine-labelled cDNA (red) from the UACC-903 cell line hybridized in the presence of poly (dA)* blocker shows little if any signal at either site (Total H = total human). Similarly, hybridization with fluorescein-labelled Cot1 DNA in the absence of competitor produces bright signal on immobilized Cot1 DNA, total human DNA and at some cDNA elements (presumed to contain highly repeated sequences, such as R23416); while Rhodamine-labelled cDNA (red) from the UACC-903 cell line produces little if any signal at these locations when hybridized in the presence of excess unlabelled poly (dA)*, and human Cot1 DNA. The absence of signal at some cDNA locations following UACC-903 cDNA hybridizations also indicates that the PCR-amplified, plasmid vector sequences at all cDNA targets do not contribute significant hybridization signal. **c**, Schematic of the array organisation. Robotic printing from 96 well microliter trays was carried out with 4 print heads, spaced to fit into 4 adjacent microliter wells. This maps the contents of each tray into four separate quadrants on the glass slide. A colour-coded map of the general distribution of target types in each of the resulting quadrants is shown.

els were elevated by the addition of a normal chromosome 6 (17 genes) are known to be activated by IFN- γ , a cardinal proinflammatory cytokine that, among other activities, induces expression of the gene products of the MHC class II locus. For example, the mRNA encoding monocyte chemotactic protein 1 (MCAF/MCP1), a cytokine that induces monocyte chemotaxis and activation^{15,16}, was more than 10-fold less abundant in the tumorigenic cell line. In the skin, MPC1 is critical in the regulation of cutaneous monocyte trafficking¹⁶⁻¹⁷, and elevated expression plays a role in suppression of tumour growth and metastasis¹⁹⁻²¹. The mechanism by which these interferon- γ regulated genes are induced in UACC-903 cells by transfer of a normal chromosome 6 remains to be determined. It is worth noting, however, that the interferon- γ receptor gene is localized to the distal long arm of human chromosome 6.

Finally, several genes that showed >10-fold higher expression in the suppressed UACC-903(+6) cells have previously been recognized in other models of tumour suppression. Most notably, there was elevated expression of the mRNA encoding WAF1 (p21), a key mediator of tumour suppression by p53 (ref. 18). The p21 protein had previously been identified as a melanoma differentiation-associated antigen (termed mda-6)^{19,20}. In melanoma cell lines suppressed for metastasis by the introduction of chromosome 6, expression of WAF1 (p21) mRNA and protein correlates inversely with metastatic potential²⁰.

These results provide a wide view of the diverse systems that are altered in this model system of tumorigenicity, and focus attention on specific gene products and pathways that may be of particular importance in this tumour type.

Our ability to classify human cancers in a way that reflects the underlying molecular pathology or that anticipates their potential for progression or response to treatment, remains primitive. Using cDNA microarrays to define alterations in gene expression associated with a specific cancer may be an efficient way to uncover clues to the specific molecular derangements that contribute to its pathogenesis and thus identify potential targets for therapeutic intervention. Moreover, recognition of pathognomonic alterations in gene expression might provide a basis for improved diagnosis and molecular classification of cancers and thus allow selection of the most appropriate therapeutic strategies.

Public databases of human expressed gene sequences contain partial sequences of at least 40,000 different human genes¹¹, and efforts to develop a human transcript map have developed rapidly²¹. Based on the high yield of information obtained using an array of <1,000 different genes, a more comprehensive survey of gene expression patterns, using a more complete array of human genes, will likely provide a rich source of new and useful insights into human biology and a deeper understanding of the gene pathways involved in the pathogenesis of cancer and other diseases.

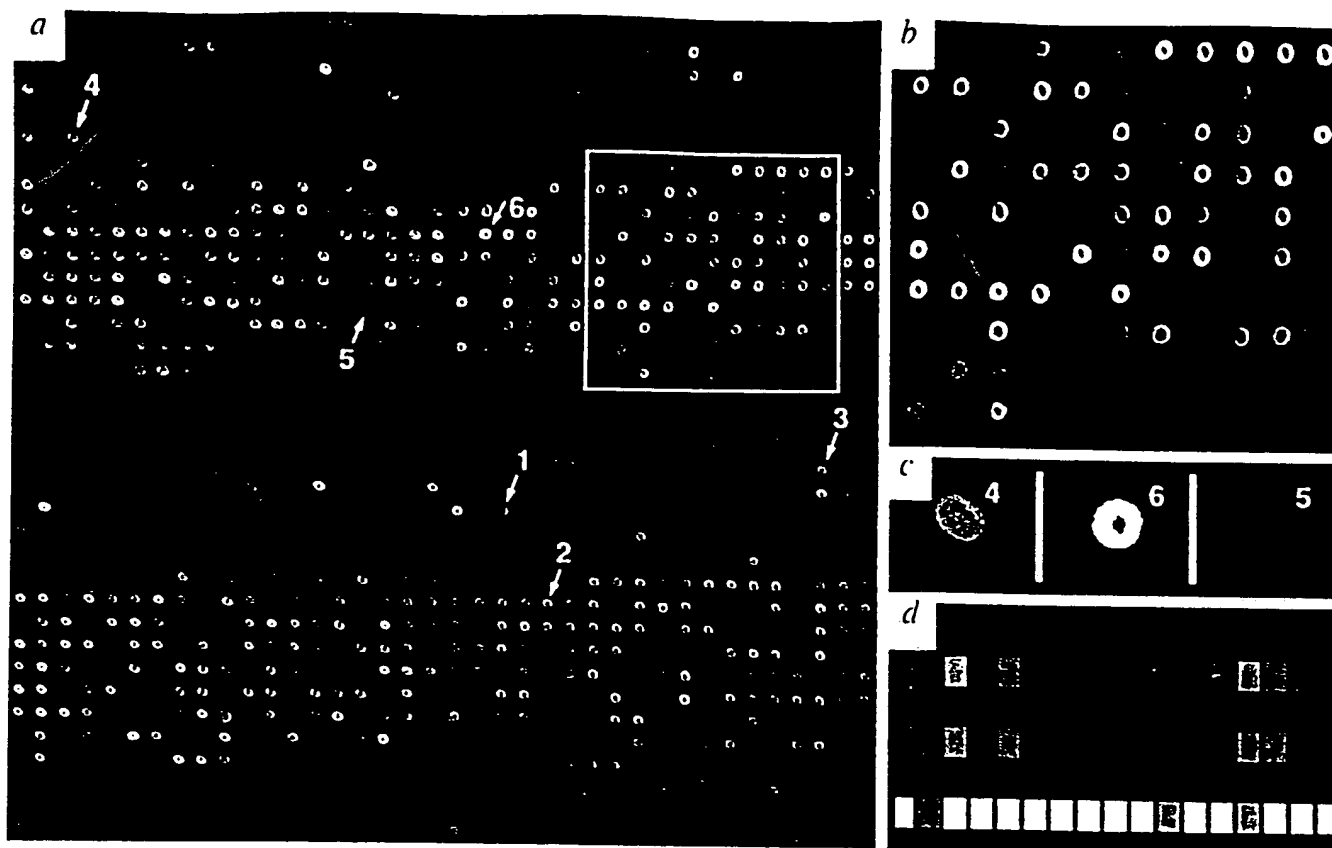


Fig. 2 DNA microarray analysis of changes in gene expression between the tumorigenic cell line, UACC-903, and its non-tumorigenic derivative, UACC903(+6), derived by introduction of a normal chromosome 6. **a**, A ratio image of the results of simultaneous hybridization of Rhodamine110-labelled cDNA (green) from UACC-903 and Cy3-labelled cDNA (orange-red) from UACC-903(+6) to a microarray. To produce this image, the scan images corresponding to each fluorescent probe were combined as the appropriate colour channels in a single image. Arrows indicate the location within the array of the corresponding genes analysed by northern blotting (Fig. 3). **b**, A magnified image of the area of the array boxed in white in (a). **c**, Magnified image of three cDNAs identified by arrows in (a), representing the cDNAs for: left, *MCAF/MCP-1* (*r/g* ratio >10); centre, *β -actin* (*r/g* ratio 1.04); and right, *α -1-antichymotrypsin* (*r/g* ratio 0.2) [see Fig. 3]. **d**, simplified representation of ratio hybridization results. Quantitative fluorescence intensity data is extracted from each array target. The average target colour ratio determines the hue of each box and the average intensity determines the brightness of each box. In this image, the order of the boxes corresponds to their original order in the microtiter plate from which they were printed. Duplicate printings of the same plate can be examined side by side, as in the first two rows shown here, to assess reproducibility of the hybridization results (see text). Numbered arrows indicate the location within the array corresponding to genes analysed by northern blotting in Fig. 3.

Methods

Generation of microarrays, hybridization, scanning. The preparation of coated microscope slides and subsequent robotic printing of DNA was carried out in a manner similar to that described⁷. Briefly, pre-cleaned glass slides were treated with poly-L-lysine solution (Sigma) to form an adhesive surface for printing. PCR products, purified by ethanol purification, were resuspended in 3x SSC. A custom built arraying robot picked up and deposited small volumes (~5 nanoliters) of DNA onto the slides. After printing, the slides were washed in a 0.2% SDS solution. The remaining bound DNA was denatured by submerging the slides in 95 °C distilled water for 2 min followed by a brief wash with 95% ethanol. DNA was UV crosslinked to the slides (Stratagene Stratilinker, 60 ml). To prevent non-specific probe binding, the slides were blocked by rinsing in a solution of 70 mM succinic anhydride dissolved in 0.1 M boric acid pH 8.0, containing 35% 1-methyl-2-pyrrolidinone (Aldrich). Additional protocols and parts list pertaining to microarray fabrication can be obtained from <http://cmgm.stanford.edu/pbrown>.

Purified, labelled cDNA was resuspended in 11 μ l of 3.5x SSC containing 4 μ g of poly (dA)⁺ DNA, 2.5 μ g *E. coli* tRNA, 4 μ g of human Cot1 DNA (Gibco BRL), and 0.3 μ l of 10% SDS. Prior to hybridization, the solution was boiled for 2 min then allowed to cool to room temperature. Hybridization was carried out at 62 °C for ~14 h in a water bath. Prior to scanning, slides were washed in 2x SSC, 0.2% SDS for 5 min and 0.2x SSC for 1 min.

Microarrays were scanned using a confocal laser scanning microscope built by S. Smith with software written

by N. Ziv. A separate scan, using the appropriate excitation line, was done for each of the two fluorophores used. Data was collected at a maximum resolution of 9 microns/pixel with 12 bits of depth.

Probe preparation and labelling. RNA was extracted from cells using the Triazol reagent (LTI Inc.), following the manufacturer's directions. cDNA probes were synthesized from singly oligo dT-selected (Pharmacia) mRNA pools. Fluorescently labelled cDNA was prepared from mRNA by oligo dT-primed polymerization using SuperScript II reverse transcriptase (LTI Inc.). The pool of nucleotides in the labelling reaction was 0.5 mM dGTP, dATP and dCTP and 0.2 mM dTTP. Fluorescent nucleotides, Rhodamine 110 dUTP (Perkin Elmer Cetus) or Cy3 dUTP (Amersham), were present at 0.1 mM. Probes were purified by gel chromatography (BioSpin 6/BioRad) and ethanol precipitation.

Selection of cDNA elements and generation of control templates. Synthetic cDNAs were prepared by cloning random *Bam*HI and *Hind*III ended fragments of *E. coli* DNA in the vector pSP64 poly (A)⁺ (Promega), linearizing isolated plasmid DNA with *Eco*RI and synthesizing poly (A)⁺ tailed RNA complementary to the insert from the resident SP6 promoter (Promega). Prior to use, the synthesized RNAs were selected on oligo dT cellulose. The largest group of cDNAs consisted of 674 cDNA clones from the INIB arrayed normalized infant brain library⁸. These clones were selected to include every INIB library member that corresponded to a named gene according

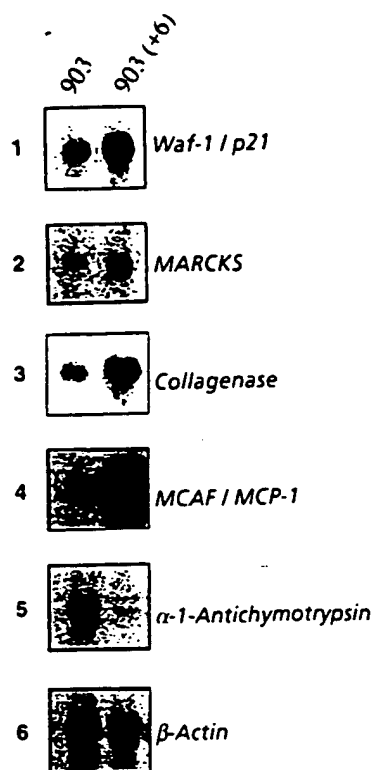


Fig. 3 Northern hybridization substantiating the consistency of the cDNA microarray results. Corresponding locations within the cDNA microarray illustrated in Fig. 2a are provided for 1) *Waf-1/p21*; 2) *MARCKS*; 3) *collagenase*; 4) *MCAF/MCP-1*; 5) α -1-*antichymotrypsin*; and 6) β -*actin*. The signal detected by a radio-labelled β -*actin* probe represents a control for loading variance, with a red/green ratio observed on the cDNA microarray (Fig. 2a,c) for β -*actin* of 1.04.

to the UniGene EST clustering system^{21,22}. The second largest group of clones consisted of 183 sequenced cDNA clones generated by subtraction of cDNA from the chromosome-6 suppressed non-tumorigenic UACC-903 (+6) cell line with cDNA from its parental tumorigenic cell line UACC-903 (ref. 9). Approximately 100 additional genes (total 870 genes arrayed) were obtained from EST libraries on the basis of their expression pattern (tissue specific, and so on). Each array included the following hybridization controls: plasmid vector, lambda, ϕ X174 phage, total human DNA, human Cot1 DNA, and poly (A)⁺. The synthetic standards used for normalization of signals in each wavelength were also arrayed. Controls were included in

each quadrant of the array to assess the reproducibility of the hybridization signal. Two plates of cDNA clones (derived from the UACC-903 subtracted library) were also arrayed in duplicate. Fidelity of the UniGene array relative to dbEST was tested by sequencing of a random sample of 11 clones used for microarray construction. All sequences were identical with the

corresponding dbEST entries. Additionally, each microarrayed cDNA from the UACC-903 subtracted library was sequenced. A listing of cDNAs comprising this microarray which were derived from the Unigene and 'housekeeping' panel can be obtained from <http://www.nih.gov/DIR/LCG/ARRAY/expn.html>.

Northern blot analysis. Total RNA, 10 μ g per lane, was electrophoresed in 1.2% agarose-formaldehyde gels and transferred onto nylon membrane (Hybond-N⁺, Amersham) by capillary blotting overnight. For DNA probes insert fragments from the Soares INIB cDNA library¹⁰ were obtained by vector PCR for p21, MARCKS, α -1-antichymotrypsin and β -actin. Probes for fibroblast collagenase and MCAF/MCP-1 were isolated from a UACC-903(+6) enriched cDNA library⁹ with all probes labelled by random priming. Filters were washed to a stringency of 0.1 \times SSC at 42 $^{\circ}$ C for 20 min.

Web sites. <http://cmgm.stanford.edu/phbrown> for protocols and parts list pertaining to microarray fabrication, <http://www.ncbgr.nih.gov/DIR/LCG/ARRAY/expn.html> for a listing of cDNAs comprising this microarray which were derived from the Unigene and 'housekeeping' panel.

Acknowledgements

Work in P.O.B.'s laboratory is supported in part by the Howard Hughes Medical Institute and National Center for Human Genome Research (HG00450). We would like to acknowledge the excellent technical and graphic assistance of X. He, T. Hofmann, Y. Jiang, J. Lenders, D. Leja and B. Walker. J.D. was supported by NIH grant 2T32BM107276-21. P.O.B. is an assistant investigator of the Howard Hughes Medical Institute.

Received 15 October; accepted 8 November, 1996.

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